Thermo SCIENTIFIC

PRODUCT INFORMATION

Thermo Scientific EpiJET DNA Methylation Analysis Kit (MspI/HpaII)

#K1441 **200 rxns**

Lot ___ **Expiry Date _**

5'...**C C G G**...3'
3'...**G G C C**...5'

Store at -20°C

www.thermoscientific.com/onebio

Kit components

Component	#K1441 200 rxns
Epi MspI	200 µL
Epi HpaII	200 µL
10X EpiBuffer	1.2 mL
Control pUC19/SmaI DNA Unmethylated (0.5 µg/µL)	20 µL
Control pUC19/SmaI DNA CpG Methylated (0.5 µg/µL)	20 µL

CERTIFICATE OF ANALYSIS

The kit is tested in Epi MspI and Epi HpaII digestion of methylated and unmethylated pUC19/SmaI DNA mixed with human blood genomic DNA. Unmethylated plasmid DNA is completely cleaved in one hour with both enzymes in presence of genomic DNA. Methylated plasmid DNA is cleaved with Epi MspI and not cleaved by Epi HpaII in presence of genomic DNA.

Quality authorized by: Jurgita Zilinskiene

Rev.5 53

Description

5-methylcytosine is a prominent epigenetic DNA modification which plays an important role in up- and down-regulation of gene expression.
The Thermo Scientific™ EpiJET™ DNA Methylation Analysis Kit (MspI/HpaII) uses a pair of restriction enzymes with different sensitivity to methylation to analyze DNA methylation status at a specific locus. Epi MspI and Epi HpaII are isoschizomers with CCGG specificity. When the internal CpG in 5'-CCGG-3' tetranucleotide is methylated, cleavage with Epi HpaII is blocked, but cleavage with Epi MspI is not affected. The Epi MspI and Epi HpaII enzymes are specially formulated for epigenetic studies to complete genomic DNA digestion in 1 hour.

Application

CpG methylation analysis at the 5'-CCGG-3' loci.

Important Notes

- To minimize the possibility of pipetting errors during the DNA digestion, prepare enough reaction master mix (including DNA sample, but not including Epi HpaII and Epi MspI) for three reactions plus 10% extra. Mix thoroughly and dispense the mixture into three sterile tubes (19 µL each). Add 1 µL of nuclease-free water into tube **1**, add 1 µL of Epi HpaII into tube **2**, add 1 µL of Epi MspI into tube **3** and follow protocol recommendations.
- For control reactions prepare two distinct reaction master mixes (one with Control pUC19/SmaI DNA Unmethylated and the other with Control pUC19/SmaI DNA CpG Methylated). Mix thoroughly and dispense each mixture into three sterile tubes (19 µL each). Add 1 µL of nuclease-free water into tubes **1** and **4**, add 1 µL of Epi HpaII into tubes **2** and **5**, add 1 µL of Epi MspI into tubes **3** and **6** and follow protocol recommendations.
- DNA sample quality is important for an efficient DNA digestion by restriction endonucleases. We recommend use of spin column based kits such as the Thermo Scientific GeneJET™ Genomic DNA Purification Kit (#K0721/2) for genomic DNA purification.
- Reactions can be scaled up, incrementally increasing reaction volume by 10 µL and enzyme amount by 1 µL for every microgram of DNA used. For example, to digest 2 µg of sample DNA use 2 µL of Epi MspI or Epi HpaII in 30 µL reaction volume. If digesting less than 1 µg, or if DNA concentration is high enough (≥125 ng/µL) to accommodate 1 µg of DNA into total volume of 8 µL, reaction volume can be scaled down to 10 µL.
- When the external C in the sequence CCGG is methylated, cleavage with both Epi MspI and Epi HpaII is blocked.
- DNA targets containing 5-hydroxymethylated cytosines are cleaved by Epi MspI, but at significantly reduced rate. We recommend extending digestion times up to 4 hours, when working with DNA isolated from 5-hmC rich tissues, like brain or stem cells.

Protocol

Read the Important Notes section before starting.

1. Mix the following components in sterile microcentrifuge tubes:

Component	Undigested DNA	Digested with Epi HpaII	Digested with Epi MspI
	1	2	3
Sample DNA	up to 1 µg*	up to 1 µg*	up to 1 µg*
10X Epi Buffer	2 µL	2 µL	2 µL
Epi HpaII	-	1 µL	-
Epi MspI	-	-	1 µL
Water, nuclease-free (#R0581)	to 20 µL	to 20 µL	to 20 µL
Total volume	20 µL	20 µL	20 µL

2. Incubate all samples at 37°C for 1 hour.
3. Terminate the reactions by incubation at 90°C for 10 min.

- To prepare protein free DNA, extract with phenol/chloroform.

*For methylation analysis by qPCR use 0.04-0.4 µg of sample DNA.

Control Reaction

- To assess DNA sample quality and potential inhibitory effects, perform control reaction using the provided unmethylated and methylated control DNA.
- Read the Important Notes section before starting.

1. Mix the following components in sterile microcentrifuge tubes:

Component	Undigested DNA	Digested with Epi HpaII	Digested with Epi MspI
	1	2	3
Sample DNA	0.5 µg	0.5 µg	0.5 µg
Control pUC19/SmaI DNA Unmethylated	0.5 µg	0.5 µg	0.5 µg
10X Epi Buffer	2 µL	2 µL	2 µL
Epi HpaII	-	1 µL	-
Epi MspI	-	-	1 µL
Water, nuclease-free (#R0581)	to 20 µL	to 20 µL	to 20 µL
Total volume	20 µL	20 µL	20 µL

Component	Undigested DNA	Digested with Epi HpaII	Digested with Epi MspI
	4	5	6
Sample DNA	0.5 µg	0.5 µg	0.5 µg
Control pUC19/SmaI DNA CpG Methylated	0.5 µg	0.5 µg	0.5 µg
10X Epi Buffer	2 µL	2 µL	2 µL
Epi HpaII	-	1 µL	-
Epi MspI	-	-	1 µL
Water, nuclease-free (#R0581)	to 20 µL	to 20 µL	to 20 µL
Total volume	20 µL	20 µL	20 µL

2. Incubate all samples at 37°C for 1 hour.
3. Terminate the reactions by incubation at 90°C for 10 min.
4. Analyze the reaction products by DNA electrophoresis on an agarose gel.

Typical results are presented in Fig. 1.
Human blood genomic DNA (uppermost band) is only partially digested by Epi HpaII and fully fragmented by Epi MspI that is not sensitive to CpG methylation. Unmethylated plasmid DNA is digested by both enzymes, while methylated plasmid DNA is susceptible only to Epi MspI digestion. Any inhibition of control DNA cleavage by restriction endonucleases indicates the presence of inhibitors. In such a case repurification of sample DNA is recommended.

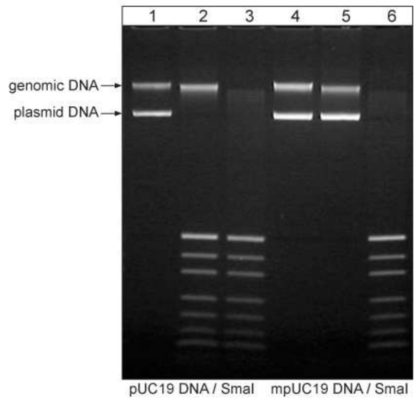


Figure 1. Digestion of genomic and control DNA by Epi MspI and Epi HpaII
Lanes 1 and 4 – undigested DNA,
Lanes 2 and 5 – digested with Epi HpaII,
Lanes 3 and 6 – digested with Epi MspI,
Lanes 1-3 contain Control pUC19/SmaI DNA Unmethylated,
Lanes 4-6 contain Control pUC19/SmaI DNA CpG Methylated.
Genomic DNA in all lanes is human blood gDNA.

Guidelines for qPCR

If CpG methylation at a particular CCGG locus is analyzed by qPCR, follow the recommendations provided below.

- For qPCR set primers that flank the CCGG site of interest.
- Use 1-2 µL (2-40 ng) of template prepared according to the Protocol and follow the manufacturer's recommendations for qPCR reaction set-up and cycling conditions.
- During qPCR setup, it is important to avoid DNA cross-contamination. We recommend using a dedicated set of pipettes for qPCR to minimize contamination.
- The accuracy of qPCR is highly dependent on accurate pipetting and thorough mixing of individual reaction components. Take extra care to avoid pipetting errors during qPCR set up and when preparing templates for qPCR. Use of 2-3 technical replicates is highly recommended.
- For calculation of 5-mC percentage use the formula provided in the Calculation of 5-mC Percentage section. To determine the PCR efficiency value prepare a standard calibration curve by diluting the "Undigested DNA" sample.

Recommendations for qPCR

- The parameters below are recommended for qPCR using Thermo Scientific Maxima™ SYBR Green/ROX qPCR Master Mix (#K0221/2/3).
- To minimize the possibility of pipetting errors, prepare a reaction master mix by adding the following components (excluding template DNA) for each 20 µL reaction to a tube at room temperature:

Maxima SYBR Green/ROX qPCR Master Mix (2X)	10 µL
Forward Primer	0.3 µM
Reverse Primer	0.3 µM
Sample DNA prepared according to the Protocol	1-2 µL (2-40 ng)
Water, nuclease-free	to 20 µL
Total volume	20 µL

- Mix the reaction master mix thoroughly and dispense the desired volume into PCR tubes or plates.
- Add template DNA (1-2 µL (2-40 ng)) prepared according to the Protocol to the individual PCR tubes or wells containing master mix. Centrifuge briefly if needed.

Note. *To minimize inaccuracies associated with pipetting smaller volumes we recommend to dilute template DNA 3-fold and use 3-6 µL.*

- Program the thermal cycler according to the recommendations below:

Step	Temperature, °C	Time	Number of Cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 sec	40
Annealing	60*	30 sec	
Extension	72	30 sec	

* use the optimal annealing temperature for your primer pair.

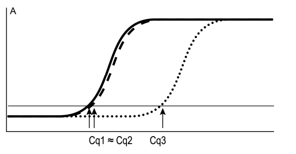
- Use two-step cycling protocol, if the optimal annealing temperature for your primer pair is 60°C:

Step	Temperature, °C	Time	Number of Cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 sec	40
Annealing/ Extension	60	60 sec	

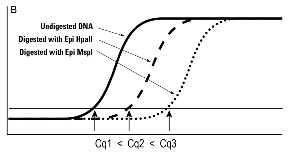
Note. *Melting curve analysis may be performed to verify the specificity of the PCR product. Primer-dimers may occur during PCR if the primer design is not optimal. The dimers are distinguished from the specific product through their lower melting point.*

Interpretation of qPCR Results

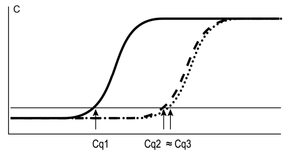
For interpretation of qPCR results see the picture provided below.



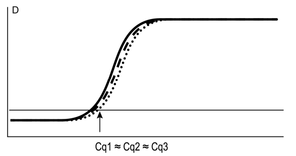
If Cq3 – Cq1 ≥ 4.5* and Cq1 ≈ Cq2** **Full methylation**. Methylation blocked the Epi HpaII cleavage. CCGG site is fully methylated.



If Cq3 – Cq1 ≥ 4.5* and Cq1 < Cq2 < Cq3 **Partial methylation**. Epi HpaII cleavage level represents methylation level in the CCGG site. Calculates into 5-mC percentage.



If Cq3 – Cq1 ≥ 4.5* and Cq2 ≈ Cq3** **No methylation**. CCGG site is not methylated. Methylation does not affect the cleavage.



Cq1≈ Cq2 ≈ Cq3** **No cleavage.** No Epi HpaII/Epi MspI recognition site or CCGG sequence is modified at external C (Epi HpaII/Epi MspI cleavage is blocked).

Cq1 - threshold cycle of "Undigested DNA" sample (continuous curve)
Cq2 - threshold cycle of "Digested with Epi HpaII" DNA sample (dashed curve)
Cq3 - threshold cycle of "Digested with Epi MspI" DNA sample (dotted curve)

* Cq3 – Cq1 < 4.5 means the incomplete digestion of sample DNA by Epi MspI. Repurify sample DNA or extend the digestion time.

** ≈ stands for ∆Cq ≤ 0.5

Note. *Modification of external C in the* ***C****CGG sequence blocks cleavage activity of Epi HpaII/Epi MspI. Levels of the external C methylation vary depending on sample type and origin.*

Calculation of 5-mC Percentage:

- If Cq3 – Cq1 ≥ 4.5, % of 5-mC is calculated using the formula below:

% of 5-mC =100 / (1 + E)$^{Cq2-Cq1}$

Where:
Cq1 is the threshold cycle of "Undigested DNA" sample
Cq2 is the threshold cycle of "Digested with Epi HpaII" sample
Cq3 is the threshold cycle of "Digested with Epi MspI" sample
E is the PCR efficiency value (%)

- % of 5-mC can not be accurately estimated when:

- Cq3 – Cq1 < 4.5.

This result demonstrates the incomplete digestion of sample DNA by Epi MspI. Repurify sample DNA or extend the digestion time. For high quality genomic DNA purification use commercially available gDNA purification kits, such as GeneJET Genomic DNA Purification Kit (#K0721/2).
The external C methylation in the CCGG sequence blocks the cleavage activity of Epi MspI. Some level of this type of methylation in the locus analyzed can also generate the result Cq3 – Cq1 < 4.5.

- Cq1 ≈ Cq2 ≈ Cq3.

This result demonstrates that there is no Epi HpaII/Epi MspI recognition site or that Epi HpaII/Epi MspI cleavage is blocked by the modification of external C in the CCGG sequence.

PRODUCT USE LIMITATION

This product is developed, designed and sold exclusively *for research purposes and in vitro use only.* The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals.
Please refer to www.thermoscientific.com/onebio for Material Safety Data Sheet of the product.